FLEXIBILITY IN GENE COEXPRESSION AT DEVELOPMENTAL AND EVOLUTIONARY TIMESCALES

Eva K Fischer[1*], Youngseok Song[2], Wen Zhou[3], Kim L Hoke[5]

[1] Department of Neurobiology, Physiology and Behavior, University of California Davis, Davis, CA 95616, USA

[2] Department of Statistics, West Virginia University, Morgantown, WV 26506, USA

[3] Department of Biostatistics, School of Global Public Health, New York University, New York, NY 10003, USA

[5] Department of Biology, Colorado State University, Fort Collins, CO 80523, USA

*Author for correspondence:

Eva K Fischer

Department of Neurobiology, Physiology and Behavior

University of California Davis

One Shields Avenue

Davis, CA 95616

ABSTRACT

The explosion of next-generation sequencing technologies has allowed researchers to move from studying single genes, to thousands of genes, and thereby to also consider the relationships within gene networks. Like others, we are interested in understanding how developmental and evolutionary forces shape the expression of individual genes, as well as the interactions among genes. To this end, we characterized the effects of genetic background and developmental environment on brain gene coexpression in two parallel, independent evolutionary lineages of Trinidadian guppies (*Poecilia reticulata*). We asked whether connectivity patterns among genes differed based on genetic background and rearing environment, and whether a gene's connectivity predicted its propensity for expression divergence. In pursuing these questions, we confronted the central challenge that standard approaches fail to control the Type I error and/or have low power in the presence of high dimensionality (i.e., large number of genes) and small sample size, as in many gene expression studies. Using our data as a case study, we detail central challenges, discuss sample size guidelines, and provide rigorous statistical approaches for exploring coexpression differences with small sample sizes. Using these approaches, we find evidence that coexpression relationships differ based on both genetic background and rearing environment. We report greater expression divergence in less connected genes and suggest this pattern may arise and be reinforced by selection.

INTRODUCTION

Genes neither act nor evolve in isolation. Rather, genes are members of physically and functionally interacting networks. The nature of these interactions influences the degree to which changes in gene sequence and gene expression influence higher-level phenotypes, and therefore the extent to which sequence and expression changes are constrained at developmental and evolutionary timescales. On the one hand, genes with many interaction partners (i.e., those that occupy central 'hub' positions in a network) may be targets of developmental switches and evolutionary selection because they most strongly influence network output and higher-level phenotypes (Chateigner *et al.*, 2020; Friedman *et al.*, 2020). Alternatively, expression changes in highly connected genes may be constrained by pleiotropic effects imposed by their many connections (Jeong *et al.*, 2001; Hahn & Kern, 2005), and developmental and evolutionary changes may therefore be more prevalent in peripheral genes that have lower connectivity and presumably lower pleiotropic loads (Kim *et al.*, 2007; Mähler *et al.*, 2017). These ideas are of long-standing interest but have historically been difficult to test because physical interaction networks were well-characterized in only very few species (e.g. protein networks in yeast (Jeong *et al.*, 2001; Hahn *et al.*, 2004; Jovelin & Phillips, 2009)) and simultaneously surveying expression in large numbers of genes was challenging, if not impossible. The proliferation of next generation sequencing technologies, and specifically RNA-sequencing (hereafter RNAseq), has removed these constraints from a technical perspective. Yet, analytical and statistical methods lag behind our ability to generate big data and – despite falling costs – the number of biological replicates remains small in many gene expression studies (Fischer et al. 2021). These factors impact network and gene coexpression analyses in particular.

Although network and coexpression analyses of RNAseq datasets remain less common than gene-wise differential expression analyses, studies in this area provide intriguing – albeit conflicting – results. Recent studies have shown that centrality in coexpression networks is negatively correlated with divergence in gene expression (Warnefors & Kaessmann, 2013; Mähler

63 *et al.*, 2017; Kuo *et al.*, 2023) as well as gene sequence evolution (Josephs *et al.*, 2017; Masalia
64 *et al.*, 2017; Harnqvist, 2021). Conversely, genes in peripheral positions show greater magnitude
65 expression divergence (Mähler *et al.*, 2017) and signatures of positive selection (Kim *et al.*, 2007).
66 Collectively, these findings suggest evolutionary constraints imposed on genes with high network
67 centrality and evolutionary flexibility in genes at the network periphery. Yet this conclusion is in
68 opposition with evidence for a bias toward changes in the expression of and selection on genes
69 with high centrality (Koubkova-Yu *et al.*, 2018; Chateigner *et al.*, 2020; Friedman *et al.*, 2020;
70 Rennison & Peichel, 2022). Evidence for changes in central genes include examples for positive
71 selection on genes with more interaction partners in human protein networks (Luisi *et al.*, 2015)
72 and high centrality in coexpression networks of genes associated with lung cancer (Wachi *et al.*,
73 2005). Together, these latter examples are more consistent with a contrasting hypothesis that
74 central genes better predict phenotypic variation and are therefore targets of selection.

75 The contradictory evidence for how gene centrality influences gene expression and
76 sequence evolvability begs the question of what leads to these opposing patterns. One eventuality
77 is that the above alternatives are not mutually exclusive, but complementary. Indeed, an extensive
78 survey of stress responses in *Arabadopsis thaliana* found expression differences associated with
79 drought stress in peripheral genes but cold stress in central genes (Des Marais *et al.*, 2017).
80 Similarly, some evidence suggests that changes in gene sequence versus gene expression may
81 be favored for physiological versus morphological traits (Warnefors & Kaessmann, 2013) and that
82 selection for sequence versus expression changes could act more strongly at central versus
83 peripheral network positions. The authors of (Hämälä *et al.*, 2020) suggest a 'goldilocks'
84 phenomenon, in which intermediate levels of pleiotropy facilitate evolution while excessive
85 pleiotropy inhibits evolution and insufficient pleiotropy makes selection inefficient (Hämälä *et al.*,
86 2020). If these seemingly contradictory observations – across taxa as well as within species – are
87 indeed driven by interactions within gene regulatory networks, then disentangling how interactions

among genes shape development and evolution requires understanding at what timescales and to what extent interactions among genes themselves change.

If the relationships among genes are flexible, then the degree of pleiotropy and its presumed consequences are not fixed. Understanding how changes in coexpression influence phenotypes is of keen interest for both basic and biomedical research (Gysi & Nowick, 2020; Stanford *et al.*, 2020). Evolutionary biologists are interested in whether and how changes in coexpression influence species' propensity for adaptation to novel and changing environments and whether changes in coexpression patterns can explain distinct genetic mechanisms underlying convergent phenotypes (Hu *et al.*, 2016; Koubkova-Yu *et al.*, 2018; Yu *et al.*, 2020b). Behavioral biologists are increasingly asking how 'rewiring' of coexpression networks drives behavioral changes in health and disease. For example, (Bloch *et al.*, 2021) found changes in coexpression associated with mating behavior in guppies, and meta-analyses implicated changes in gene coexpression patterns associated with behavioral disorders in humans (Gaiteri *et al.*, 2014). Changes in coexpression patterns are also of broader biomedical interest as they have been documented as a feature of diverse cancers (Wachi *et al.*, 2005; Anglani *et al.*, 2014).

The above studies highlight both the interest in and the potential of coexpression analyses. This demand is being met by a growing collection of tools for (differential) gene coexpression analysis (Wang *et al.*, 2017; Chowdhury *et al.*, 2020; Tommasini & Fogel, 2023). Yet while these software packages make advanced network analyses accessible, they do not eliminate the statistical limitations of these approaches. These limitations arise primarily from the combination of small sample sizes and high-dimensional data (tens of thousands of genes) emblematic of transcriptomic studies. While sample sizes have increased as sequencing costs have decreased, per group sample sizes commonly remain less than ten. Pooling samples across experimental groups or multiple studies can bring the total experimental sample size into the range recommended for network analyses (e.g., N=20 by (Langfelder & Horvath, 2008; Ballouz *et al.*, 2015)). However, when explicitly asking if coexpression relationships differ between two or among

114 a few experimental groups, small per group samples remain a problem. In brief, inference from
115 coexpression analyses is problematic without sufficient sample sizes and power, but also when
116 samples are pooled across experimental groups that can differ in coexpression structure. This
117 leaves researchers trapped between an experimental rock and hard place.

118 Like others, we are interested in using transcriptomic analyses to understand the biological
119 basis of complex phenotypes, and specifically in exploring changes in individual genes as well as
120 the interactions among genes. To this end, we characterized the effects of genetic background
121 (high-predation versus low-predation populations) and developmental environment (rearing with
122 and without predator chemical cues) on brain gene coexpression in two parallel, independent
123 evolutionary lineages of Trinidadian guppies (*Poecilia reticulata*). In Trinidad, downstream, high-
124 predation fish have repeatedly and independently colonized upstream, low-predation
125 environments (Gilliam *et al.*, 1993; Barson *et al.*, 2009; Willing *et al.*, 2010; Fraser *et al.*, 2015),
126 leading to parallel adaptive changes in life-history, morphology, and behavior (Reznick *et al.*,
127 1990, 2001; Endler, 1995; Reznick, 1997; Magurran, 2005). In other words, each river drainage
128 represents a naturally replicated experiment demonstrating parallel phenotypic adaptation.
129 Recent studies have used laboratory breeding designs to disentangle genetic from environmental
130 influences, demonstrating that both evolutionary history with predators and developmental
131 experience with predators shape life history (Torres Dowdall *et al.*, 2012), morphology (Torres-
132 Dowdal *et al.*, 2012; Fischer *et al.*, 2013; Ruell *et al.*, 2013; Handelsman *et al.*, 2014), physiology
133 (Handelsman *et al.*, 2013; Fischer *et al.*, 2014), and behavior (Huizinga *et al.*, 2009; Torres-
134 Dowdall *et al.*, 2012; Fischer *et al.*, 2016b). Using this breeding (Figure 1), we asked (1) whether
135 connectivity patterns among genes differed among groups, and (2) whether connectivity
136 influenced a gene's propensity for expression divergence.

137 We previously demonstrated genetic and developmental differences in brain gene
138 expression (Fischer *et al.*, 2021). Here, we were interested in testing the hypothesis that – in
139 addition to expression changes in single genes – the relationships among genes are targets of

140 developmental and evolutionary processes. In pursuing these questions, we confronted the
141 statistical challenges that standard approaches may fail to control Type I error and/or have low
142 power when dimensionality is high (i.e., large number of genes) and sample size is small. From
143 a biological perspective, we find evidence for flexibility in coexpression relationships based on
144 both genetic background and rearing environment, suggesting that changes in the interactions
145 among genes are associated with phenotypic divergence at developmental and evolutionary
146 timescales. From a technical perspective, we present a case study for those interested in
147 (differential) coexpression with small per group sample sizes. We discuss key challenges, set
148 clear sample size guidelines to control Type I error while maintaining power, and provide rigorous
149 statistical approaches for exploring coexpression differences even with small sample sizes that
150 can be readily implemented for similar coexpression analyses in other studies.
151
152
153 **RESULTS**
154 *Preliminary analyses*
155 Guppies used in this study were second generation lab-born fish from unique family lines
156 established from wild-caught high-predation (HP) and low-predation (LP) populations in the Aripo
157 and Quare river drainages in the Northern Range mountains of Trinidad. At birth, we split second-
158 generation siblings into rearing environments with (pred+) or without (pred-) predator chemical
159 cues, and they remained in these environments until the completion of the experiment (Figure 1)
160 (as in (Fischer *et al.*, 2016b)). In brief, each drainage therefore consists of a 2x2 factorial design
161 that distinguishes genetic from developmental effects of predation. Pair-wise comparisons of
162 biological relevant are: (1) HP pred- vs LP pred-, an experiment comparing populations reared in
163 an environment lacking predator cues to identify genetic differences between populations; (2) HP
164 pred+ vs HP pred-, to identify environmentally induced changes mimicking the situation in which
165 high-predation fish colonize low-predation environments, i.e., “ancestral plasticity”; (3) LP pred-

166 vs LP pred+, to identify environmentally induced changes comparable to the situation in which
167 low-predation fish are washed downstream and a measure of whether ancestral plasticity is
168 maintained in the derived population; (4) HP pred+ vs LP pred+, to identify genetic differences
169 when fish are raised with environmental cues of predation. We also compared the same
170 experimental groups across drainages (e.g. HP pred+ in Aripo drainage vs HP pred+ in Quare
171 drainage) to understand differences associated with parallel, independent evolutionary lineages.
172
173 Our initial approach was to characterize and explore gene coexpression using the popular
174 Weighted Gene Correlation Network Analysis (WGCNA) package in R (Langfelder & Horvath,
175 2008). We calculated module preservation scores using the methods implements in WGCNA
176 which combine a number of difference preservation statistics to calculate a summary preservation
177 score (Langfelder *et al.*, 2011). We found that, in both drainages, ~50% of gene coexpression
178 modules were not preserved across experimental groups (Supplemental Materials A). In other
179 words, we identified substantial differences in network structure between groups, suggesting that
180 the common practice of reconstructing coexpression networks by pooling samples across groups
181 may not be valid. In brief, our preliminary analyses using WGCNA underscored the need for a
182 statistical method to discern network differences across groups when dealing with small group
183 sizes (N=10-12 in our case). We sought to address these issues through the alternative statistical
184 approaches detailed in the Methods and Supplemental Materials B. We present results from the
185 most promising approaches below.
186

187

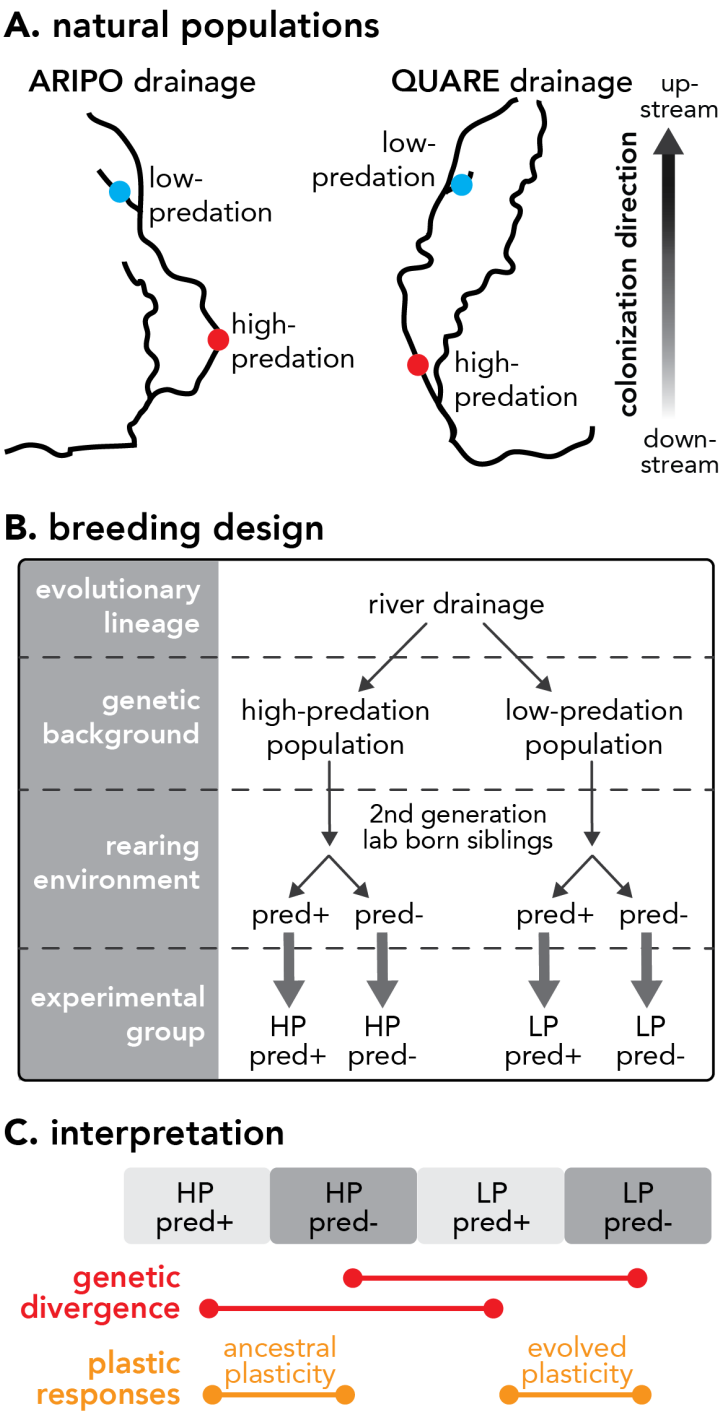


Figure 1. Conceptual overview and interpretation. (A) Natural populations in different river drainages represent distinct evolutionary lineages. (B) Overview of laboratory breeding design disentangling genetic and environmental effects. (C) Interpretation of comparisons of interest between experimental groups resulting from 2x2 breeding design shown in (B). HP = high-predation, LP = low-predation, pred+ = reared with predator chemical cues, pred- = reared without predator chemical cues. Modified from Fischer et al. 2021.

188 *Changes in coexpression networks based on genetics and environment*

189 To overcome problems associated with very small per group sample sizes, yet high-dimensional
190 data, we used random projection-based tests to compare covariance structures between
191 experimental groups. We considered the set of all genes (DE and non-DE) that passed filtering
192 criteria (Aripo: 13,446; Quare: 14,379). We found significant differences in covariance structures
193 between high-predation and low-predation fish reared with predators (HP pred+ vs LP pred+) in
194 both drainages (Figure 2; Table 1). Analysis of the Quare dataset found a marginally significant
195 difference between high-predation fish reared with and without predators (HP pred+ vs HP pred).
196 We also compared the covariance structures between the same treatment groups across
197 drainages. Here, we found significant differences in all comparisons (Table 2). In short, when
198 considering the collection of all genes, we found evidence for changes in gene coexpression
199 based on evolutionary lineage (drainage), genetic background (population), and rearing
200 environment.

201

Table 1. Approximated p-values from random projection tests comparing covariance structure for all genes (DE and non-DE) between treatment groups.

	ARIPO drainage	**QUARE drainage**
HP pred+ vs HP pred-	1.0000	0.0639
HP pred+ vs LP pred+	0.0149	<0.0001
HP pred- vs LP pred -	1.0000	0.9568
LP pred+ vs LP pred-	0.1258	0.9943

202

Table 2. Approximated p-values from random projection tests comparing covariance structure across drainages.

ARIPO vs QUARE	**p-value**
HP pred-	<0.0001
HP pred+	0.0001
LP pred -	0.0001
LP pred+	0.0107

203

204

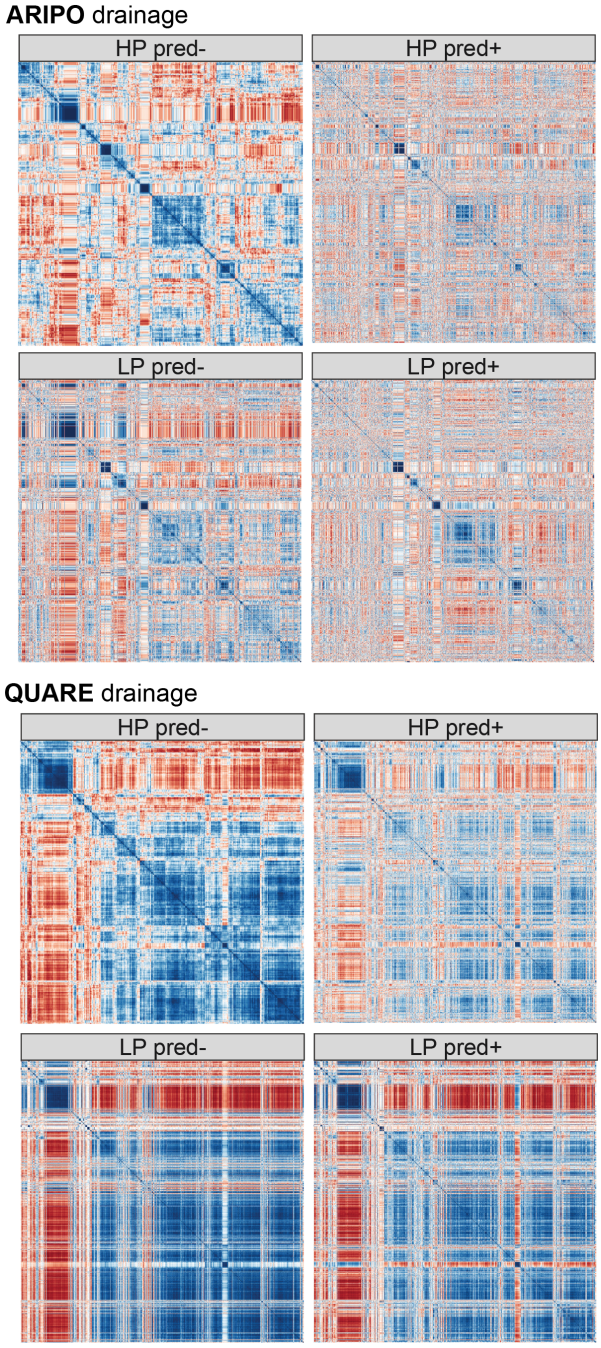


Figure 2. Visualization of coexpression differences between experimental groups and evolutionary lineages. Correlation heatmaps provide a visual approximation of statistical differences based on genetic background (high- vs low-predation), rearing environment (with (pred+) or without (pred-) predators), and evolutionary lineage (Aripo and Quare drainage). Gene order is determined by hierarchical clustering of the high-predation pred- group, meaning that the same position in two heatmaps represents the correlation of identical pairs of genes. For ease of visualization and computation, only the 1,000 most variable genes are shown.

205 *Coexpression networks and differential expression*

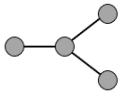
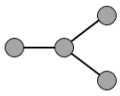
206 In addition to changes in coexpression among genes, we were interested in how interactions
207 among genes might interface with a gene's propensity to be differentially expressed at
208 developmental and evolutionary timescales. To explore this question, we compared coexpression
209 networks between differentially expressed (DE) and non-differentially expressed (non-DE) genes.
210 We performed these comparisons separately for each treatment group, given group-level
211 differences in covariance structures (see above) and DE status being inherently related to
212 differences in gene expression among treatment groups.

213 The comparison involved two steps: (i) reconstruction of the coexpression network using
214 gene-wise correlations, and (ii) comparing two networks of different sizes. To achieve (i), we first
215 tested whether the correlation between each pair of genes was zero, while controlling the FDR
216 using the method from (Cai & Liu, 2016), as detailed in Supplemental Section 4.3. Using this
217 method, an edge is drawn between any two genes (nodes) with nonzero correlation, forming the
218 coexpression network. To assess the constructed network's sensitivity to different FDR levels, we
219 compared network summary plots at multiple FDR cutoffs (α = 0.01, 0.05, 0.1). If two networks
220 are distinct, their summary plots will differ (Maugis *et al.*, 2017). The network summary plots
221 (Figure S6) suggest that the correlation-based coexpression network is relatively insensitive to
222 different FDR levels. Therefore, we used a coexpression network with α = 0.05 for all subsequent
223 analyses.

224 A major concern in comparing DE versus non-DE networks, which has been largely
225 overlooked in literature, is that the different collections of genes in these two sets (e.g., DE genes
226 are by definition a small subset of all genes) making the two corresponding networks have
227 different numbers of unmatchable nodes [60]. To address this, we adopted the network
228 comparison test proposed by (Shao *et al.*, 2022), which accommodates networks of different sizes
229 by analyzing their network moments for specific motifs using the difference of two subgraph
230 densities adjusted for their edge densities. Specifically, we used the v-shape (subgraphs with

three nodes and two edges), triangle (subgraphs with three nodes and three edges), and 3-star (subgraphs with four nodes and three edges) (see visualizations in Figure S9, Table 3) to compare the correlation-based coexpression networks. In network sciences, these subgraphs reflect connectivity and clusterability [60, 61, 62]. Apart from HP pred+ and HP pred- in the Aripo dataset, we observe that all non-DE networks had higher subgraph densities than their DE network counterparts (Figure S7 & S8; Table S8). Following this overall trend, we compared the different subgraph types described above. The Aripo dataset had fewer overall differences, especially for the 3-star subgraph type (Table S9). In the Quare dataset, DE versus non-DE networks differed in nearly all groups for all subgraph types (Table S9). To further compare subgraph densities in DE and non-DE networks, we conducted the same test with one-sided alternatives that DE gene network subgraphs are more or less connected than non-DE gene subgraphs. The results show that in the Aripo drainage, non-DE networks generally have higher sparsity-adjusted v-shape densities, while in the Quare drainage, the DE network in HPP has higher adjusted v-shape and triangle densities, but DE networks in LPP and LPNP have lower adjusted triangle and 3-star densities than their NDE counterparts (Table 3).

Table 3. Network comparisons of DE vs non-DE genes within treatment groups and across datasets. Comparisons of sparsity-adjusted subgraph densities tested the alternatives that DE gene networks had smaller or larger subgraph density than non-DE networks. P-values from one-sided alternative tests are reported for the v-shape, triangle, and 3-star subgraph types.

	ARIPO drainage			**QUARE drainage**		
	v-shape	triangle	3-star	v-shape	triangle	3-star
						
DE < non-DE						
HP pred-	0.0051	0.8611	0.6149	<0.0001	0.9523	1.0000
HP pred+	0.0676	0.2330	0.5027	<0.0001	<0.0001	0.0665
LP pred -	0.0056	0.1700	0.2650	1.0000	1.0000	1.0000
LP pred+	<0.0001	0.0152	0.0749	0.4844	1.0000	1.0000
DE > non-DE						
HP pred-	0.9948	0.1389	0.3742	1.0000	0.0482	<0.0001
HP pred+	0.9358	0.7676	0.5013	1.0000	1.0000	0.9347
LP pred -	0.9938	0.8277	0.7304	<0.0001	<0.0001	<0.0001
LP pred+	1.0000	0.9850	0.9252	0.5122	<0.0001	<0.0001

DISCUSSION

Our goal in this study was to understand how genetic background and rearing environment shape relationships among genes. We previously characterized expression changes at the level of individual genes (Fischer *et al.*, 2021), and here we were interested in exploring changes in coexpression patterns among genes. Our findings suggest that coexpression patterns are flexible at evolutionary and developmental timescales. Exciting from a biological perspective, exploring these questions presents statistical challenges, in particular for RNAseq datasets characterized by high-dimensionality and small sample sizes. We discuss the implications of our work from both angles.

Gene expression studies remain plagued by small per-group samples sizes and high dimensionality. Network construction is far from trivial, if not problematic, under these conditions, especially when network structure – and not just network expression level – differs among experimental groups. In our own study, we had an overall sample size of N=98 individuals, well

261 above the recommendation of N=30 for network construction. However, this total sample size
262 includes samples from two drainages and four experimental groups, and – based on our analyses
263 here and preliminary analyses using the WGCNA package – we found evidence that network
264 structure differs between experimental groups and even more strongly between drainages. These
265 differences are of key biological interest as they suggest that expression relationships among
266 genes (i.e., network structure) are subject to developmental plasticity and evolutionary change.
267 However, if network structure differs across experimental groups, then networks must be
268 constructed separately for each experimental group to avoid construction of 'average' networks
269 that can obscure differences of (biological) interest and lead to biased conclusions (Zhao *et al.*,
270 2014; Shojaie, 2021; Li *et al.*, 2022; Sai Li & Li, 2023). To take an extreme example, if two genes
271 have opposing correlations of the same magnitude in two groups, the average correlation across
272 groups will be zero. Thus, it is the per-group sample size that is most important for network
273 construction and comparison when gene coexpression patterns are of interest.

274 While our per-group sample size of N=10-15 is relatively large for an RNAseq study, it is
275 below the recommended threshold for network construction, such as the minimum sample of
276 N=20 suggested for RNAseq analyses by (Ballouz *et al.*, 2015). As we illustrate in the
277 Supplemental Materials, these sample sizes are surprisingly inadequate for recently developed
278 high-dimensional statistical tests thought to be robust against high-dimensionality, to control Type
279 I error, and to maintain power. Indeed, from our simulation experiments, most common methods
280 require N>50 to retain the generally accepted nominal significance levels of 0.05 and satisfactory
281 power exceeding 0.8. Importantly, the potential misinterpretations resulting from these
282 shortcomings are not systematic (i.e., directionally biased) and therefore difficult to predict. As a
283 growing number of studies consider how interactions among genes shape phenotypic differences
284 across timescales, we present our work as a case study to increase awareness of these
285 limitations, present complementary statistical approaches to those commonly used, and in hopes
286 that others will consider these issues in experimental design and analysis.

287 We first investigated whether gene coexpression patterns differed based on genetic
288 background and rearing environment. Using pairwise expression covariance tests, we identified
289 differences in coexpression patterns between fish reared with and without predators, fish adapted
290 to high- versus low-predation environments, and fish from distinct evolutionary lineages.
291 Differences in coexpression structures in both drainages were most pronounced between high-
292 and low-predation fish reared with predators (HP pred+ vs LP pred+). This comparison represents
293 the ancestral population adapted to life with predators (HP pred+) versus the derived low-
294 predation population adapted to predator and suddenly re-exposed to predator cues (e.g., as
295 when fish are washed downstream; LP pred+). Fish adapted to a low-predation life are expected
296 to be poorly equipped deal with the sudden stressors of predation. Indeed, we previously found
297 HP pred+ fish to be behaviorally least variable and LP pred+ fish to be behaviorally most variable
298 (Fischer *et al.*, 2016b). In light of findings here, we suggest that these unpredictable behavioral
299 patterns could arise from disruptions in gene coexpression networks in the brain.

300 In addition to differences within drainages, we found more evidence for coexpression
301 differences in the Quare as compared to the Aripo drainage, and that differences were ubiquitous
302 when comparing between the two drainages. We suggest these patterns arise – at least in part –
303 from the extent of genetic divergence between populations: high- and low-predation populations
304 in the Quare drainage show greater genetic (Willing *et al.*, 2010) and gene expression (Fischer *et*
305 *al.*, 2021) divergence than those in the Aripo drainage, and the two drainages represent distinct
306 evolutionary lineages (Willing *et al.*, 2010). The importance of genetic background in shaping
307 evolutionary trajectories is highlighted by our previous work demonstrating distinct underlying
308 mechanisms associated with parallel phenotypic adaptation in guppies from distinct evolutionary
309 lineages (Fischer *et al.*, 2016a, 2021). Similar mechanistic flexibility has also been demonstrated
310 in other systems (Cordero *et al.*, 2018; Jacobs *et al.*, 2020), including those known for parallel
311 phenotypic evolution (e.g. (Laporte *et al.*, 2015; Hanson *et al.*, 2017; Bolnick *et al.*, 2018)). Our
312 findings here extend these observations from the expression of individual genes to coexpression

313 patterns among genes, suggesting that alternative gene expression network configurations can
314 give rise to shared organism-level phenotypes.

315 What are the potential consequences of changes in gene coexpression relationships at
316 developmental and evolutionary timescales? If pleiotropic interactions among genes constrain
317 their propensity for change, then flexibility in these relationships could reduce pleiotropic load
318 (e.g., (Wang *et al.*, 2010; Pavlicev & Wagner, 2012; Pavličev & Cheverud, 2015). In other words,
319 genes may be more able to change in expression and drive phenotypic change if their interactions
320 with other genes can be altered to minimize off-target effects. Conversely, flexibility in
321 coexpression relationships might improve the ability of underlying gene expression networks to
322 buffer higher-level phenotypes through homeostatic change (e.g., (Fischer *et al.*, 2016a; Badyaev,
323 2018; Hoke *et al.*, 2019)). Importantly, either scenario implies that the relationships among genes
324 may themselves be targets of selection. Alternatively, changes in gene coexpression could
325 represent transcriptional noise if these changes do not amount to selectable differences at the
326 network and/or organismal level. While this last scenario is less interesting from an adaptationist
327 perspective, such 'neutral' changes may nonetheless have consequences for evolutionary
328 trajectories, for example by giving rise to cryptic variation that is revealed under novel
329 environmental conditions (West-Eberhard, 2003; McGuigan & Sgrò, 2009; Paaby & Rockman,
330 2014). In brief, all three alternatives highlight that coexpression relationships can change at
331 developmental and evolutionary timescales with consequences in the short and long term.

332 To begin to address the above alternatives, we asked whether connectivity influenced a
333 gene's propensity for expression divergence. This question has been considered at various levels,
334 including in protein-protein interactions (Kim *et al.*, 2007; Luisi *et al.*, 2015), gene expression
335 networks (Oldham *et al.*, 2006; Bloch *et al.*, 2021), gene sequence changes (Josephs *et al.*, 2017;
336 Makinen *et al.*, 2018), and the relationship between expression and sequence divergence
337 (Harrison *et al.*, 2012; Warnefors & Kaessmann, 2013; Chateigner *et al.*, 2020). Overall, we found
338 that networks of non-DE genes were denser than networks of DE genes. This suggests that DE

339 genes are less well-connected than their non-DE counterparts, supporting the idea that differential
340 expression may be facilitated by lower connectivity and decrease pleiotropy. Comparing across
341 lineages, we observed more widespread differences in network connectivity in the Quare as
342 compared to the Aripo drainage. As high- and low-predation populations in the Quare drainage
343 are more genetically diverged than those in the Aripo (Willing *et al.*, 2010), this observations
344 suggests that greater genetic divergence may increase network sparseness, suggesting that
345 selection can reshape network structure. Taken together, our findings support greater expression
346 divergence in less connected genes and suggest this pattern could facilitate changes in network
347 topology if there is feedback between greater magnitude expression change and decreased
348 network connectivity.
349
350 *Conclusions*
351 Understanding how underlying genetic architecture shapes the maintenance and evolution of
352 complex traits is a fundamental goal of biological research. Over the past two decades, the
353 explosion of next-generation sequencing technologies has allowed us to move beyond the genetic
354 scale – considering one or a few genes or loci – to genomic scales – considering thousands to
355 tens of thousands of genes or loci. Among the key advances afforded by these approaches, are
356 the ease of conducing broadscale, exploratory studies; the opportunity to characterize underlying
357 mechanisms in non-model species; and the ability to consider genes in the context of their
358 interactions. As a growing number of studies consider how interactions among genes shape
359 phenotypic differences across timescales, we provide a case study to increase awareness of
360 limitations and provide suggestions for analysis. Our findings provide intriguing evidence of
361 extensive coexpression flexibility at multiple timescales in a species known for rapid adaptation.
362 The generality of these phenomena and their consequences for adaptation will be revealed by
363 more studies with larger sample sizes and new statistical approaches. Understanding whether
364 and how relationships among genes change at developmental and evolutionary timescales has

365 consequences for our understanding of how underlying mechanisms shape flexibility and
366 robustness in higher order phenotypes, how animals adapt to novel and changing environments,
367 and how behavior and physiology are regulated in health and disease.
368
369
370 **METHODS**
371 *Fish collection and rearing*
372 Samples here are the same as those described in (Fischer *et al.*, 2021). Briefly, guppies used in
373 this study were second generation lab-born fish from unique family lines established from wild-
374 caught high-predation (HP) and low-predation (LP) populations in the Aripo and Quare river
375 drainages in the Northern Range mountains of Trinidad. At birth, we split second-generation
376 siblings into rearing environments with (pred+) or without (pred-) predator chemical cues, and
377 they remained in these environments until the completion of the experiment (Figure 1) (as in
378 (Fischer *et al.*, 2016b)). Guppies were individually housed in 12:12 hour light cycle and fed a
379 measured food diet once daily. All experimental methods were approved by the Colorado State
380 University Animal Care and Use Committee (Approval #12-3818A).
381
382 *Tissue collection and processing*
383 We collected brain tissue from mature males in the groups described above 10 minutes after
384 lights on in the morning. We extracted RNA from whole brains using the Qiagen RNeasy Lipid
385 Tissue Mini Kit (Qiagen, Germany) and constructed a sequencing library for each individual using
386 the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Massachusetts,
387 USA). For the Aripo dataset, 40 samples (N=10 per group) were pooled with unique barcodes
388 into eight samples per sequencing library and each library was sequenced on a single lane. For
389 the Quare dataset, 60 samples (N=12-16 per group) were pooled into three sequencing libraries
390 with 20 samples per pool and each library was sequenced in two separate lanes. Libraries were

391 sequenced as 100bp paired-end reads on an Illumina HiSeq 2000 at the Florida State University
392 College of Medicine Translational Science Laboratory (Tallahassee, Florida) in May 2014 (Aripo
393 dataset) and January 2016 (Quare dataset).
394
395 *Differential expression analysis*
396 We reported results of differential expression analyses in another study (Fischer *et al.*, 2021), and
397 we use the resulting differential expression status (DE versus non-DE) as an important criterion
398 in analyses performed here (see below). Briefly, we normalized read counts using DESeq2 (Love
399 *et al.*, 2014) and performed differential expression analysis using the lme4 package in R
400 (github.com/lme4). We used generalized linear mixed models with a negative binomial link to
401 accommodate our experimental design and data type, including family and sampling week as
402 random effects. Population of origin (HP / LP), rearing environment (pred+ / pred-), and their
403 interaction were included as fixed effects. We used the residuals in the covariance and correlation
404 analyses described below to adjust for fixed and random effects included in model fitting. To
405 compare differentially expressed and non-differentially expressed genes, we adjusted p-values
406 for multiple hypothesis testing using a direct approach for FDR control (Storey, 2002) as
407 implemented in the fdrtool package in R (Strimmer, 2008). We considered transcripts differentially
408 expressed (DE) if the adjusted p-value was <0.05, and all other genes non differentially expressed
409 (non-DE).
410
411 *Challenges from small sample, high dimensional data*
412 In wanting to understand differences in covariance structure and changes in network architecture
413 between our experimental groups, we faced two fundamental challenges. First, controlling Type
414 I error becomes difficult when comparing large networks or covariance structures with limited
415 sample sizes, often leading to spurious discoveries and unreliable results. Also, detecting true
416 effects for separating complicated networks or covariance structures in ultra-high dimensional

417 datasets is difficult due to low statistical power; a common phenomenon in coexpression analysis
418 of RNAseq studies. While there are commonly accepted methods for two sample covariance
419 tests, they either fail to control the Type I error rate with extremely small sample size (e.g., N<25
420 per group) or have substantially low power (see Supplemental Materials for simulations) (Li &
421 Chen, 2012; Cai *et al.*, 2013; Chang *et al.*, 2017; Yu *et al.*, 2020a). Second, comparison of multiple
422 networks is nontrivial, particularly when the networks are of different sizes or have unmatching
423 nodes (Tang *et al.*, 2017; Agterberg *et al.*, 2020; Qi *et al.*, 2024). For gene coexpression analysis
424 these issues apply, for example, when sample sizes vary between groups and are fairly small due
425 to the limitation of experiment constraints, or when comparing subsets of genes of interest that
426 vary in size (e.g., comparing non-DE vs DE gene sets, or differently sized coexpression modules)
427 (Agterberg *et al.*, 2020; Alyakin *et al.*, 2024; Jin *et al.*, 2024; Qi *et al.*, 2024).

428 To highlight these challenges, we first present simulation studies using existing high-
429 dimensional methods designed for valid inference on comparing large covariance structures with
430 controlled Type I error rate and reasonable power (Li & Chen, 2012; Cai *et al.*, 2013; Chang *et*
431 *al.*, 2017; Yu *et al.*, 2020a), before presenting our method and examining the real data set (see
432 below). We summarize the outcomes of the simulation studies and exploratory comparisons here
433 and refer the interested reader to additional details provided in the Supplemental Information.

434 We found that Type I error rates for existing tests were uncontrolled for small sample sizes
435 of N<50 per group, even when the number of genes was relatively small (250 genes, orders of
436 magnitude smaller than what is typical for RNAseq analysis) (Figure S1). In addition to
437 uncontrolled Type I error, the existing methods were substantially underpowered for small sample
438 sizes. Specifically, the empirical power was overall low (<0.25) for sample sizes N<30 per group
439 (Figure S2). These issues plagued our dataset, which is representative of most RNAseq studies
440 exploring connections between gene expression and behavior (per group samples N<10,
441 ~20,000+ genes). Importantly, these issues are not resolved by subsampling the data to include
442 a smaller number of genes (Figure S3), an approached commonly deployed by network analysis

443 packages (e.g., filtering for the 5,000-8,000 most variable genes in WGCNA or the approach of
444 (Qiu *et al.*, 2021)).
445
446 *Our new high-dimensional covariance comparison*
447 From the above simulations, it is clear that existing approaches to compare large covariance
448 structures fail even when using only a small subset of genes. To overcome these issues, we
449 employed the random projection (Wu & Li, 2020) to develop a new two-sample covariance
450 comparison method (Supplemental Information). This new test can control Type I error (Figure
451 S4) and have satisfactory power (Figure S5), even with very small per group sample sizes and a
452 large number of genes.
453 We conduct our newly developed random projection-based tests on residual from the
454 generalized linear mixed model described above and in (Fischer *et al.*, 2021). We focused on the
455 pairwise comparisons of biological interest (Figure 1c). Within each drainage, we compared (1)
456 HP pred- vs LP pred-, an experiment comparing populations reared in an environment lacking
457 predator cues to identify genetic differences between populations; (2) HP pred+ vs HP pred-, to
458 identify environmentally induced changes mimicking the situation in which high-predation fish
459 colonize low-predation environments, i.e., “ancestral plasticity”; (3) LP pred- vs LP pred+, to
460 identify environmentally induced changes comparable to the situation in which low-predation fish
461 are washed downstream and a measure of whether ancestral plasticity is maintained in the
462 derived population; (4) HP pred+ vs LP pred+, to identify genetic differences when fish are raised
463 with environmental cues of predation. We also compared the same experimental groups across
464 drainages (e.g. HP pred+ in Aripo drainage vs HP pred+ in Quare drainage) to understand
465 differences associated with parallel, independent evolutionary lineages. For both within and
466 between drainage comparison, we considered the four comparisons jointly to control family-wise
467 error rate.
468

Correlation network comparisons

We used correlation network analyses to examine whether DE versus non-DE genes differed in their relationships with other genes. A challenge with this analysis was the lack of consensus to define networks, in addition to the fact that derived networks will usually have very different sizes and unmatched nodes (i.e., non-DE genes far outnumber DE genes and a single gene is inherently only in one category). We constructed adjacency matrices using the correlations of residuals from the aforementioned generalized linear mixed model. From these adjacency matrices, we identified overall network structure and the frequency of specific subgraphs (i.e., small motifs or subnetworks with two to seven nodes and one to six edges) for each experimental group within each drainage. We then compared network pairs of interest to examine their structural differences, using a network summary plot and two-sample network tests based on relative frequencies of different subgraphs adjusted for the sparsity of networks (additional details in Supplemental Information) (Maugis *et al.*, 2017; Shao *et al.*, 2022).

ACKNOWLEDGEMENTS

We thank the members of the Colorado State University Guppy Group for fish care and help with tissue collection and processing. This work was supported by the National Science Foundation DDIG-1311680 (to EKF), RCN IOS-1256839 (to EKF), IOS-1354755 (to KLH), IOS 1922701 (to WZ) , US Department of Energy DE-SC0018344 (to WZ), and NIH R01GM144961 (to WZ).

DATA ACCESSIBILITY

Raw sequencing reads are available through the NCBI SRA repository (PRJNA601479). R code for statistical analyses will be made available on GitHub (https://github.com/EnigmaSong/GeneFlexibilityStudy) upon publication.

AUTHOR CONTRIBUTIONS

EKF and KLH conceived of the study; EKF collected samples and performed molecular work, gene expression mapping, transcript abundance estimation, and preliminary differential expression analyses; YS and WZ devised and performed statistical analyses with input from EKF and KLH; EKF and YS performed data visualization; EKF wrote the manuscript with contributions from all authors.

REFERENCES

Agterberg, J., Tang, M. & Priebe, C.E. 2020. Nonparametric two-sample hypothesis testing for random graphs with negative and repeated eigenvalues. *arXiv.* 2012.09828.

Alyakin, A.A., Agterberg, J., Helm, H.S. & Priebe, C.E. 2024. Correcting a Nonparametric Two-sample Graph Hypothesis Test for Graphs with Different Numbers of Vertices with Applications to Connectomics. *Appl Netw Sci.* 9: 1.

Anglani, R., Creanza, T.M., Liuzzi, V.C., Piepoli, A., Panza, A., Andriulli, A., *et al.* 2014. Loss of connectivity in cancer co-expression networks. *PLoS One.* 9: 0087075.

Badyaev, A. V. 2018. Evoulutionary transitions in controls reconcile adaptation with continuity of evolution. *Semin Cell Dev Biol.* 88: 36–45.

Ballouz, S., Verleyen, W. & Gillis, J. 2015. Guidance for RNA-seq co-expression network construction and analysis: Safety in numbers. *Bioinformatics.* 31: 2123–2130.

Barson, N.J., Cable, J. & Van Oosterhout, C. 2009. Population genetic analysis of microsatellite variation of guppies (*Poecilia reticulata*) in Trinidad and Tobago: Evidence for a dynamic source-sink metapopulation structure, founder events and population bottlenecks. *J Evol Biol.* 22: 485–497.

521 Bloch, N.I., Corral-López, A., Buechel, S.D., Kotrschal, A., Kolm, N. & Mank, J.E. 2021. Different
522 mating contexts lead to extensive rewiring of female brain coexpression networks in the
523 guppy. *Genes Brain Behav.* 20: 1–11.
524 Bolnick, D.I., Barrett, R.D.H., Oke, K.B., Rennison, D.J. & Stuart, Y.E. 2018. (Non)Parallel
525 evolution. *Annu Rev Ecol Evol Syst.* 49: 303–330.
526 Cai, T., Liu, W. & Xia, Y. 2013. Two-sample covariance matrix testing and support recovery in
527 high-dimensional and sparse settings. *J Am Stat Assoc.* 108: 265–277.
528 Cai, T.T. & Liu, W. 2016. Large-Scale Multiple Testing of Correlations. *J Am Stat Assoc.* 111:
529 229–240.
530 Chang, J., Zhou, W., Zhou, W.X. & Wang, L. 2017. Comparing large covariance matrices under
531 weak conditions on the dependence structure and its application to gene clustering.
532 *Biometrics.* 73: 31–41.
533 Chateigner, A., Lesage-Descauses, M.C., Rogier, O., Jorge, V., Leplé, J.C., Brunaud, V., *et al.*
534 2020. Gene expression predictions and networks in natural populations supports the
535 omnigenic theory. *BMC Genomics.* 21: 1–16.
536 Chowdhury, H.A., Bhattacharyya, D.K. & Kalita, J.K. 2020. (Differential) Co-Expression Analysis
537 of Gene Expression: A Survey of Best Practices. *IEEE/ACM Trans Comput Biol Bioinform.*
538 17: 1154–1173.
539 Cordero, G.A., Liu, H., Wimalanathan, K., Weber, R., Quinteros, K. & Janzen, F.J. 2018. Gene
540 network variation and alternative paths to convergent evolution in turtles. *Evol Dev.* 20:
541 172–185.
542 Des Marais, D.L., Guerrero, R.F., Lasky, J.R. & Scarpino, S. V. 2017. Topological features of a
543 gene co-expression network predict patterns of natural diversity in environmental response.
544 *Proceedings of the Royal Society B: Biological Sciences.* 284: 20170914.
545 Endler, J.A. 1995. Multiple-trait coevolution and environmental gradients in guppies. *Trends*
546 *Ecol Evol* .10: 22–29.

547 Fischer, E.K., Ghalambor, C.K. & Hoke, K.L. 2016a. Can a Network Approach Resolve How
548 Adaptive vs Nonadaptive Plasticity Impacts Evolutionary Trajectories? *Integr Comp Biol.*
549 56: 877–888.
550 Fischer, E.K., Ghalambor, C.K. & Hoke, K.L. 2016b. Plasticity and evolution in correlated suites
551 of traits. *J Evol Biol.* 29: 991–1002.
552 Fischer, E.K., Harris, R.M., Hofmann, H.A. & Hoke, K.L. 2014. Predator exposure alters stress
553 physiology in guppies across timescales. *Horm Behav.* 65: 165–172.
554 Fischer, E.K., Hauber, M.E. & Bell, A.M. 2021a. Back to the basics? Transcriptomics offers
555 integrative insights into the role of space, time and the environment for gene expression
556 and behaviour. *Biology Letters*. 2021;17:20210293.
557 Fischer, E.K., Soares, D., Archer, K.R., Ghalambor, C.K. & Hoke, K.L. 2013. Genetically and
558 environmentally mediated divergence in lateral line morphology in the Trinidadian guppy
559 (*Poecilia reticulata*). *Journal of Experimental Biology.* 216: 3132–3142.
560 Fischer, E.K., Song, Y., Hughes, K.A., Zhou, W. & Hoke, K.L. 2021. Non-parallel transcriptional
561 divergence during parallel adaptation. *Mol Ecol.* 30: 1516–1530.
562 Fraser, B.A., Künstner, A., Reznick, D.N., Dreyer, C. & Weigel, D. 2015. Population genomics of
563 natural and experimental populations of guppies (*Poecilia reticulata*). *Mol Ecol.* 24: 389–
564 408.
565 Friedman, D.A., York, R.A., Hilliard, A.T. & Gordon, D.M. 2020. Gene expression variation in the
566 brains of harvester ant foragers is associated with collective behavior. *Commun Biol.* 3.
567 Springer US.
568 Gaiteri, C., Ding, Y., French, B., Tseng, G.C. & Sibille, E. 2014. Beyond modules and hubs: The
569 potential of gene coexpression networks for investigating molecular mechanisms of
570 complex brain disorders. *Genes Brain Behav.* 13: 13–24.
571 Gilliam, J.F., Fraser, D.F. & Alkins-Koo, M. 1993. Structure of a Tropical Stream Fish
572 Community : A Role for Biotic Interactions. *Ecology.* 74: 1856–1870.

573 Gysi, D.M. & Nowick, K. 2020. Construction, comparison and evolution of networks in life
574 sciences and other disciplines. *J R Soc Interface.* 17.
575 Hahn, M.W., Conant, G.C. & Wagner, A. 2004. Molecular Evolution in Large Genetic Networks:
576 Does Connectivity Equal Constraint? *J Mol Evol.* 58: 203–211.
577 Hahn, M.W. & Kern, A.D. 2005. Comparative genomics of centrality and essentiality in three
578 eukaryotic protein-interaction networks. *Mol Biol Evol.* 22: 803–806.
579 Hämälä, T., Gorton, A.J., Moeller, D.A. & Tiffin, P. 2020. Pleiotropy facilitates local adaptation to
580 distant optima in common ragweed (*Ambrosia artemisiifolia*). *PLoS Gene.t* 16: 1–23.
581 Handelsman, C.A., Broder, E.D., Dalton, C.M., Ruell, E.W., Myrick, C.A., Reznick, D.N., *et al.*
582 2013. Predator-induced phenotypic plasticity in metabolism and rate of growth: Rapid
583 adaptation to a novel environment. *Integr Comp Biol.* 53: 975–988.
584 Handelsman, C.A., Ruell, E.W., Torres-Dowdall, J. & Ghalambor, C.K. 2014. Phenotypic
585 Plasticity Changes Correlations of Traits Following Experimental Introductions of
586 Trinidadian Guppies (*Poecilia reticulata*). *Integr Comp Biol.* 54: 794–804.
587 Hanson, D., Hu, J., Hendry, A.P. & Barrett, R.D.H. 2017. Heritable gene expression differences
588 between lake and stream stickleback include both parallel and antiparallel components.
589 *Heredity.* 119: 339–348.
590 Harnqvist SE, Grace CA, Jeffares DC. Variables Influencing Differences in Sequence
591 Conservation in the Fission Yeast *Schizosaccharomyces pombe*. *J Mol Evol*. 2021;89.
592 Harrison, P.W., Wright, A.E. & Mank, J.E. 2012. The evolution of gene expression and the
593 transcriptome-phenotype relationship. *Semin Cell Dev Biol.* 23: 222–229.
594 Hoke, K.L., Adkins-Regan, E., Bass, A.H., McCune, A.R. & Wolfner, M.F. 2019. Co-opting evo-
595 devo concepts for new insights into mechanisms of behavioural diversity. *Journal of*
596 *Experimental Biology.* 222: jeb190058.

597 Hu, G., Hovav, R., Grover, C.E., Faigenboim-Doron, A., Kadmon, N., Page, J.T., *et al.* 2016.
598 Evolutionary conservation and divergence of gene coexpression networks in *Gossypium*
599 (Cotton) seeds. *Genome Biol Evol.* 8: 3765–3783.
600 Huizinga, M., Ghalambor, C.K. & Reznick, D.N. 2009. The genetic and environmental basis of
601 adaptive differences in shoaling behaviour among populations of Trinidadian guppies,
602 *Poecilia reticulata*. *J Evol Biol.* 22: 1860–1866.
603 Jacobs A, Carruthers M, Yurchenko A, Gordeeva N V., Alekseyev SS, Hooker O, et al.
604 Parallelism in eco-morphology and gene expression despite variable evolutionary and
605 genomic backgrounds in a Holarctic fish. *PLoS Genetics*. 2020;16:e1008658.
606 Jeong, H., Mason, S.P., Barabási, A.L. & Oltvai, Z.N. 2001. Lethality and centrality in protein
607 networks. *Nature.* 411: 41–42.
608 Jin, X., Chan, K., Barnett, I. & Ghosh, R.P. 2024. Two-Sample Hypothesis Testing for Large
609 Random Graphs of Unequal Size. *arXiv.* 2024.11133.
610 Josephs, E.B., Wright, S.I., Stinchcombe, J.R. & Schoen, D.J. 2017. The relationship between
611 selection, network connectivity, and regulatory variation within a population of *Capsella*
612 *grandiflora*. *Genome Biol Evol.* 9: 1099–1109.
613 Jovelin, R. & Phillips, P.C. 2009. Evolutionary rates and centrality in the yeast gene regulatory
614 network. *Genome Biol.* 10:R35.
615 Kim, P.M., Korbel, J.O. & Gerstein, M.B. 2007. Positive selection at the protein network
616 periphery: Evaluation in terms of structural constraints and cellular context. *Proc Natl Acad*
617 *Sci.* 104: 20274–20279.
618 Koubkova-Yu, T.C.T., Chao, J.C. & Leu, J.Y. 2018. Heterologous Hsp90 promotes phenotypic
619 diversity through network evolution. *PLoS Biol.* 16: e2006450.
620 Kuo, H.C., Yao, C. Te, Liao, B.Y., Weng, M.P., Dong, F., Hsu, Y.C., *et al.* 2023. Weak gene–
621 gene interaction facilitates the evolution of gene expression plasticity. *BMC Biol.* 21: 1–20.

622 Langfelder, P. & Horvath, S. 2008. WGCNA: An R package for weighted correlation network
623 analysis. *BMC Bioinformatics.* 9: 1–13.
624 Langfelder, P., Luo, R., Oldham, M.C. & Horvath, S. 2011. Is my network module preserved and
625 reproducible? *PLoS Comput Biol.* 7: e1001057.
626 Laporte, M., Rogers, S.M., Dion-Côté, A.M., Normandeau, E., Gagnaire, P.A., Dalziel, A.C., *et*
627 *al.* 2015. RAD-QTL mapping reveals both genome-level parallelism and different genetic
628 architecture underlying the evolution of body shape in lake Whitefish (*Coregonus*
629 *clupeaformis*) species pairs. *G3: Genes, Genomes, Genetics.* 5: 1481–1491.
630 Li, J. & Chen, S.X. 2012. Two sample tests for high-dimensional covariance matrices. *Ann Stat.*
631 40: 908–940.
632 Li, S., Cai, T.T. & Li, H. 2022. Transfer Learning in Large-Scale Gaussian Graphical Models with
633 False Discovery Rate Control. *J Am Stat Assoc*. 118: 2171–2183.
634 Love, M.I., Huber, W. & Anders, S. 2014. Moderated estimation of fold change and dispersion
635 for RNA-seq data with DESeq2. *Genome Bio.l* 15: 1–21.
636 Luisi, P., Alvarez-Ponce, D., Pybus, M., Fares, M.A., Bertranpetit, J. & Laayouni, H. 2015.
637 Recent positive selection has acted on genes encoding proteins with more interactions
638 within the whole human interactome. *Genome Biol Evol.* 7: 1141–1154.
639 Magurran, A.E. 2005. *Evolutionary ecology: the Trinidadian guppy*. Oxford University Press,
640 Oxford.
641 Mähler, N., Wang, J., Terebieniec, B.K., Ingvarsson, P.K., Street, N.R. & Hvidsten, T.R. 2017.
642 Gene co-expression network connectivity is an important determinant of selective
643 constraint. *PLoS Genet.* 13: 1–33.
644 Makinen, H., Savilammi, T., Papakostas, S., Leder, E., Vøllestad, L.A. & Primmer, C.R. 2018.
645 Modularity facilitates flexible tuning of plastic and evolutionary gene expression responses
646 during early divergence. *Genome Biol Evol.* 10: 77–93.

Masalia, R.R., Bewick, A.J. & Burke, J.M. 2017. Connectivity in gene coexpression networks negatively correlates with rates of molecular evolution in flowering plants. *PLoS One* 12: 1–10.

Maugis, P.-A.G., Olhede, S.C. & Wolfe, P.J. 2017. Topology Reveals Universal Features for Network Comparison. *arXiv.*1705.05677.

McGuigan, K. & Sgrò, C.M. 2009. Evolutionary consequences of cryptic genetic variation. *Trends Ecol Evol.* 24: 305–311.

Oldham, M.C., Horvath, S. & Geschwind, D.H. 2006. Conservation and evolution of gene coexpression networks in human and chimpanzee brains. *Proc Natl Acad Sci.* 103: 17973–17978.

Paaby, A.B. & Rockman, M. V. 2014. Cryptic genetic variation: Evolution's hidden substrate. *Nat Rev Genet.* 15: 247–258.

Pavličev, M. & Cheverud, J.M. 2015. Constraints Evolve: Context Dependency of Gene Effects Allows Evolution of Pleiotropy. *Annu Rev Ecol Evol. Syst* 46: 413–434.

Pavlicev, M. & Wagner, G.P. 2012. A model of developmental evolution: Selection, pleiotropy and compensation. *Trends Ecol Evol.* 27: 316–322.

Qi, M., Li, T. & Zhou, W. 2024. Multivariate Inference of Network Moments by Subsampling. *arXiv.* 2409.01599.

Qiu, T., Xu, W. & Zhu, L. 2021. Two-sample test in high dimensions through random selection. *Comput Stat Data Anal.* 160: 107218.

Rennison, D.J. & Peichel, C.L. 2022. Pleiotropy facilitates parallel adaptation in sticklebacks. *Mol Ecol.* 31: 1476–1486.

Reznick, D., Butler IV, M.J. & Rodd, H. 2001. Life-History Evolution in Guppies. VII. The Comparative Ecology of High- and Low-Predation Environments. *Am Nat.* 157: 126–140.

Reznick, D.A., Bryga, H. & Endler, J.A. 1990. Experimentally induced life-history evolution in a natural population. *Nature.* 346: 357–359.

673 Reznick, D.N. 1997. Life history evolution in guppies (*Poecilia reticulata*): Guppies as a model
674 for studying the evolutionary biology of aging. *Exp Gerontol.* 32: 245–258.
675 Ruell, E.W., Handelsman, C.A., Hawkins, C.L., Sofaer, H.R., Ghalambor, C.K. & Angeloni, L.
676 2013. Fear, food and sexual ornamentation: plasticity of colour development in Trinidadian
677 guppies. *Proceedings of the Royal Society B: Biological Sciences.* 280: 20122019–
678 20122019.
679 Sai Li, T.T.C. & Li, H. 2023. Transfer Learning in Large-Scale Gaussian Graphical Models with
680 False Discovery Rate Control. *J Am Stat Assoc.* 118: 2171–2183.
681 Shao, M., Xia, D., Zhang, Y., Wu, Q. & Chen, S. 2022. Higher-Order Accurate Two-Sample
682 Network Inference and Network Hashing. *arXiv*. 2208.07573.
683 Shojaie, A. 2021. Differential network analysis: A statistical perspective. *Wiley Interdisciplinary*
684 *Reviews: Computational Statistics*. 2021;13:e1508.
685 Stanford, B.C.M., Clake, D.J., Morris, M.R.J. & Rogers, S.M. 2020. The power and limitations of
686 gene expression pathway analyses toward predicting population response to
687 environmental stressors. *Evol Appl.* 13: 1166–1182.
688 Storey, J.D. 2002. A direct approach to false discovery rates. *J R Stat Soc Series B Stat*
689 *Methodol.* 64: 479–498.
690 Strimmer, K. 2008. fdrtool: A versatile R package for estimating local and tail area-based false
691 discovery rates. *Bioinformatics.* 24: 1461–1462.
692 Tang, M., Athreya, A., Sussman, D.L., Lyzinski, V., Park, Y. & Priebe, C.E. 2017. A
693 Semiparametric Two-Sample Hypothesis Testing Problem for Random Graphs. *Journal of*
694 *Computational and Graphical Statistics.* 26: 344-354.
695 Tommasini, D. & Fogel, B.L. 2023. multiWGCNA: an R package for deep mining gene co-
696 expression networks in multi-trait expression data. *BMC Bioinformatics.* 24: 1–15.

697 Torres Dowdall, J., Handelsman, C.A., Ruell, E.W., Auer, S.K., Reznick, D.N. & Ghalambor,
698 C.K. 2012. Fine-scale local adaptation in life histories along a continuous environmental
699 gradient in Trinidadian guppies. *Funct Ecol.* 26: 616–627.
700 Torres-Dowdall, J., Handelsman, C. a, Reznick, D.N. & Ghalambor, C.K. 2012. Local adaptation
701 and the evolution of of phenotypic plasticity in Trinidadian guppies (*Poecilia reticulata*).
702 *Evolution.* 66: 3432–3443.
703 Torres-Dowdall, J., Handelsman, C.A., Reznick, D.N. & Ghalambor, C.K. 2012. Local adaptation
704 and the evolution of phenotypic plasticity in Trinidadian guppies (*Poecilia reticulata*).
705 *Evolution.* 66: 3432–3443.
706 Wachi, S., Yoneda, K. & Wu, R. 2005. Interactome-transcriptome analysis reveals the high
707 centrality of genes differentially expressed in lung cancer tissues. *Bioinformatics.* 21: 4205–
708 4208.
709 Wang, D., Wang, J., Jiang, Y., Liang, Y. & Xu, D. 2017. BFDCA: A Comprehensive Tool of
710 Using Bayes Factor for Differential Co-Expression Analysis. *J Mol Biol.* 429: 446–453.
711 Wang, Z., Liao, B.-Y. & Zhang, J. 2010. Genomic patterns of pleiotropy and the evolution of
712 complexity. *Proceedings of the National Academy of Sciences.* 107: 18034–18039.
713 Warnefors, M. & Kaessmann, H. 2013. Evolution of the correlation between expression
714 divergence and protein divergence in mammals. *Genome Biol Evol.* 5: 1324–1335.
715 West-Eberhard, M.J. 2003. *Developmental plasticity and evolution*. Oxford University Press,
716 New York.
717 Willing, E.M., Bentzen, P., Van Oosterhout, C., Hoffmann, M., Cable, J., Breden, F., *et al.* 2010.
718 Genome-wide single nucleotide polymorphisms reveal population history and adaptive
719 divergence in wild guppies. *Mol Ecol.* 19: 968–984.
720 Wu, T.-L. & Li, P. 2020. Projected Tests for High-Dimensional Covariance Matrices. *J Stat Plan*
721 *Inference.* 207: 73–85.

722 Yu, X., Li, D. & Xue, L. 2020a. Fisher's Combined Probability Test for High-Dimensional
723 Covariance Matrices. *arXiv.* 2006.00426.
724 Yu, Y., Hu, H., Doust, A.N. & Kellogg, E.A. 2020b. Divergent gene expression networks underlie
725 morphological diversity of abscission zones in grasses. *New Phytologist* 225: 1799–1815.
726 Zhao, S.D., Cai, T.T. & Li, H. 2014. Direct estimation of differential networks. *Biometrika.* 101:
727 253–268.
728